

The Development of a Typing System for Pseudomonas aeruginosa

An Honors Thesis (HONRS 499)

by

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A handwritten signature in cursive script, reading "J. R. Garcia", is written over a horizontal dashed line.

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Abstract

Pseudomonas aeruginosa is an important opportunistic pathogen, although many of its epidemiologic questions remain unanswered. In order to understand the epidemiology a valid typing method must be used to accurately distinguish strains of *Pseudomonas aeruginosa*. An epidemiological marker should possess three characteristics; yield the same results on repeated typing (reproducibility), be discriminating to strain differences (discrimination), and yield a small number of non-typeable strains (typability). Southern analysis using DNA probes is a technique recently developed to type the different strains which may fit these criteria. This study has been designed to compare this method with the other typing methods available. Although the results from the other laboratories are not yet available for comparison, our results have shown seventeen strains out of two hundred and twenty three isolates which exhibit undistinguishable probe reactive fragments. Other typing method's results will be compared to the DNA probes in order to determine a standard method of classification. This standard will be used to answer various questions concerning the epidemiology and pathogenicity of the organism.

Introduction

Pseudomonas aeruginosa was first cultured from a clinical infection in 1882. By the end of the 19th century it had been cultured from nearly every anatomic site of infection. However, it did not emerge as a significant human pathogen until the last three decades. Later studies demonstrated that *P. aeruginosa* can be recovered from stool cultures in 6-10% of the healthy, general population but, these organisms have little tendency to invade these hosts (5). Patients prone to infection include immunocompromised (especially neutropenic) patients, and those patients with malignancy, cystic fibrosis, burns, or traumatic wounds. Studies also illustrated that *P. aeruginosa* is responsible for a large number of nosocomial infection in the United States with only *E. coli*, and *S. aureus* being more common (5). One reason *P. aeruginosa* causes a large number of nosocomial infections is because it is resistant to many commonly used antibiotics. In addition, *P. aeruginosa* is metabolically diverse and can adapt to harsh environments. For example, *P. aeruginosa* can survive on sixty different organic compounds including disinfectants found in hospitals. These properties have made *P. aeruginosa* an important pathogen.

Many important questions remain unanswered regarding the pathogenicity and epidemiology of *P. aeruginosa*. For example, are environmental strains as virulent to patients as those strains contracted through cross-infection? How frequently does cross-infection occur among patients with cystic fibrosis? Additionally, will identical strains exhibit identical or similar pathological features in different patients? For these reasons and many others, a reliable typing scheme is important in differentiating strains of *P. aeruginosa*.

An ideal typing system would yield the same result on repeated typing (reproducibility), would be discriminating to strain differences (discrimination), and would yield a small number of non-typable strains (typability). In the past, several different typing systems have been used for epidemiological investigations in attempt to answer some of the above questions, including : antimicrobial susceptibility profiles (3,4), pyocin sensitivity or production (3,4,7,8), sensitivity to

bacteriophage lysis (2,4) and, O-serotyping (11,22,25,27). More recent techniques include genome fingerprinting using pulsed field electrophoresis (4), and Southern analysis with labelled DNA probes (15). Each method has disadvantages as well as advantages in terms of reproducibility, typability, and discrimination among different isolates.

Pseudomonas aeruginosa may undergo dissociation, a biological phenomenon that results in daughter colonies having characteristics different from those of the original colony (4). Dissociation can occur in vitro and in vivo and contributes to the difficulty of typing *P.*

aeruginosa using the typing schemes mentioned above. For example, phage susceptibility testing of colonial dissociants often yields variable results. Antimicrobial susceptibility profiles done on parental isolates and on progeny may also differ significantly. Other problems occur with the presently accepted typing systems. For example, 50-70% of isolates of *P. aeruginosa* cultured from patients with cystic fibrosis are non-typable or polyagglutinate in O-typing sera, (10,16,19). Also, infection of the organism with bacteriophages may cause a change in the O-serotype. The changes in both phage type and antimicrobial susceptibility patterns indicate that these methods are not based on a stable genetic characteristics. The problem arises when typing is based on an expressed phenotype such as LPS, flagella, or antimicrobial susceptibility profiles. The solution to this problem may be in a genetically based typing system. Several DNA typing methods have been recently developed which could alleviate the problem associated with methods based on phenotypic properties. Some of these methods include genomic fingerprinting by pulse field electrophoresis, dot blot hybridization with a pilin probe, and Southern blot analysis using probes derived from the exotoxin A and adjacent 5' sequences. Additional discussion of these methods will follow.

This study is designed to compare and contrast the various methods of typing *P. aeruginosa* in terms of typability, reproducibility and discrimination among different isolates. The objective is to obtain a method which is efficient, cost effective, and, most importantly, accurate. Through the use of this investigation a standard method may

be established in order to answer the ever important epidemiological questions being raised.

Materials and Methods

Typing Methods. The investigation was designed as a multicenter, blinded study to compare different methods for typing *Pseudomonas aeruginosa*. The methods which will be compared are: serotyping with the International Antigenic Typing Serum (IATS) reagents supplied by Difco (6), serotyping with monoclonal anti-LPS reagents (11,22,25,27), pyocin production typing (3,4,7,8), multilocus enzyme electrophoresis (23), dot blot hybridization with a pilin gene probe (18,21), phage typing (2,4), genome fingerprinting by pulse field electrophoresis (4), and Southern blot analysis using probes derived from exotoxin A and adjacent 5' sequences (15,26). Each typing scheme will be evaluated according to its percentage of typable strains, reproducibility, and discrimination among different isolates. A strain is considered untypable if it cannot be typed on any replicate, weakly typable if it can be typed on at least one replicate and strongly typable if it can be typed on all replicates. Reproducibility is only relevant with respect to strongly typable strains. An assay is considered reproducible with respect to the given strain if it yields the same result on all replicates of that strain, and unreproducible if the assay does not give identical types on replicates. Discrimination is defined with respect to strains that are reproducible. In this case, an assay is discriminating with respect to two strains if types for the two strains are distinct. Each investigator was blind to the origin and identity of each isolate.

Bacterial Strains. The *P. aeruginosa* cultures used in this study were obtained from the collection of David P. Speert, M.D. at the University of British Columbia, Vancouver, B.C. Canada. One-hundred and fifty *P. aeruginosa* isolates from patients with cystic fibrosis were selected for the study based on known typing results; seventy-five were monotypable and seventy-five were nontypable or polytypable with International Antigenic Typing Serum (IATS) reagents. The isolates were selected to represent as many different serotypes, pilin gene RFLPs and enzyme allotypes as possible. Seventeen *P. aeruginosa* isolates which are the Difco prototype strains used for the production of the IATS reagents and known to possess different LPS serotypes

were also used in the study. Ten environmental *P. aeruginosa* isolates were obtained from cultures of river water, sink drains or vegetables. Twenty-three additional clinical isolates from patients without cystic fibrosis were also typed. Each of 200 separate *P. aeruginosa* isolates were typed on three separate occasions by each participating investigator. Isolates were supplied after coding one through six hundred.

Southern Analysis Using Probes Derived From Exotoxin A and Adjacent 5' Sequences. The methods used for Southern blot analysis, which was the technique used in this laboratory, are detailed below.

Isolation of Chromosomal DNA. Isolates were grown in 30 mls of Brain-heart infusion broth (Difco Laboratories, Detroit, MI) and shaken overnight at 37° C. The DNA was extracted using the method of Marmur (13) after lysis of the bacteria by the method of Hirt (12).

Quantification of Isolated DNA. UV spectrophotometric readings at wavelengths of 260 nm and 280 nm were used to estimate DNA and protein concentrations. The reading at 260 nm allows estimation of concentration of nucleic acid in the sample (which will be utilized in preparation of the enzymatic digests). The ratio of the reading at 260/280 nm provides an estimate of the purity of the nucleic acid preparation. Protein contamination of the sample is evident if the ratio is significantly less than 1.8.

Digestion with Restriction Endonucleases. Each DNA sample was individually digested using restriction endonucleases *Sal I* which cuts at 5'-G TCGA C-3', *Bgl II* which cuts at 5'-A GATC T-3', and *Xho I* which cuts at 5'-C TCGA G-3'. 3 ug of the purified DNA was digested with the above listed restriction endonucleases using the manufactures' instructions (Bethesda Research Laboratories, Life Technologies, Inc. Gaithersburg, MD).

Southern Hybridization. 0.5-1.0 ug of digested DNA was electrophoresed through a 0.6% agarose gel at 40 V for ~18 hours. Each sample contained 10 ul of a mixture containing 1X Tris Acetate Buffer (TAE), 50% glycerol and 0.05 g bromophenol blue, and 5 ul of the digested DNA in water. DNA samples were transferred onto nitrocellulose paper (Nytran Nucleic Acid and Protein Transfer Media, Schleicher & Schuell, Keene, NH) using the method of Southern (24).

— DNA was fixed to nitrocellulose by baking for 2 hours in a vacuum oven at 80° C, or using UV irradiation (120,000 microjoules), (Stratalinker UV Crosslinker 1800 and 2400, Stratagene, Inc., LaJolla, CA). The hybridization solution contained 50% (vol/vol) formamide, 1 M sodium chloride, 10% dextran sulfate, and 1% SDS.

— Purification and Characterization of DNA Probes. The probe used for Southern hybridization was either a 741 bp *PstI-NruI* fragment or a 2730 bp *Pst I-EcoRI* fragment which includes the 5' region of the exotoxin A structural gene and 5' and structural gene sequences respectively. Both probes were cloned from strain PA 103 (26). *PstI-NruI* was used for all digestions with *XhoI*, and *PstI-EcoRI* was used for *Sall* and *BglII*. The probes were labeled with dCT³²P by nick-translation using commercially available kits (Amersham, Arlington Heights, III). Salmon sperm DNA at a final concentration of 200 ug/ml was included in each hybridization. Labeled probe and salmon sperm DNA were heat denatured at 95° C for 5 minutes. The nitrocellulose membranes were prehybridized in the hybridization solution for ~6 hours, then hybridized for 18 hr at 42° C after addition of the probe. Nitrocellulose membranes were washed for 45 minutes at 65° C with 5 ml/cm² 5X Sodium Chloride/Sodium Citrate (SSC), 0.1% SDS, then soaked for 15 minutes each in 1 x SSC, 0.5-1.0% at 37° C.

— Autoradiographs. Nitrocellulose membranes were exposed to X-Omat AR film (Eastman Kodak Rochester, NY) for ~24 hours at -70° C in autoradiograph cassettes.

— Typing. For each isolate typed the molecular weight of probe reactive *Sall*, *BglII*, and *XhoI* fragments was assigned by comparison to molecular weight standards included on each gel. Isolates were also compared by visual inspection of ethidium bromide stained agarose gels. Molecular weight standards used were lambda phage DNA digested with *Hind III* and a 1 Kb DNA Ladder (BRL, Gaithersburg, MD). Isolates were considered from the same strain when restriction fragments identified on the Southern blots were identical in size. Isolates were considered from different strains when one or more fragments differed in molecular weight using any of the three restriction enzymes. The error in assigning molecular weights by this means was determined by repetitive typing of fragments of known size

within the range of molecular weights of interest, and is estimated as 5% for fragments <10 Kb and 10% for fragments >10 Kb (J. Ogle, unpublished observations).

Results

Typing results are completed for 223/600 isolates, and are shown on Table 1. Results for 63 isolates are incomplete for one or more of the three restriction endonucleases. 57 of 160 isolates, for which data is complete, are divided into 17 groups as shown in Table 2. Typing results on isolates within each of the 17 groups are not demonstrably different. For this preliminary analysis, strains were considered undistinguishable only when two of the three endonuclease fragments were exact and the other varied only by 0.1.

Table 1. Summary of Southern Hybridization Results:

Strain	<i>Bgl</i> II*	<i>Sal</i> I*	<i>Xho</i> I*
1	5.1	10.1	6.3
2	7.4	3.6	2.8
3	9.9	11.0	13.0
4	10.3	11.8	8.8
5	5.0	6.2	6.3
6	5.0	10.1	6.3
7	9.2	9.4	+
8	12.2	+	+
9	5.0	7.6	2.8
10	7.6	9.4	11.8
11	8.1	4.0	6.1
12	8.1	4.0	6.1
13	8.1	4.0	6.1
14	8.7	9.4	12.2
15	+	3.7	2.6
16	8.5	3.9	6.2
17	5.0	9.6	6.4
18	7.7	+	5.2
19	8.5	3.9	6.2
20	7.4	8.4	10.5
21	11.5	13.8	15.5
22	5.2	7.2	6.4
23	8.8	4.1	6.2
24	5.2	10.2	6.4
25	8.8	4.1	6.2
26	9.8	3.8	5.2
27	10.8	12.2	8.8
28	4.8	10.8	9.1
29	7.7	3.8	5.2
30	10.5	11.5	8.2
31	5.0	10.2	+
32	7.4	3.5	2.8
33	9.0	11.5	6.7
34	9.4	9.4	14.5
35	8.5	4.0	6.2
36	8.5	4.0	6.2
37	4.9	9.6	6.4
38	11.2	10.2	+
39	7.4	3.7	2.7

Strain	<i>Bgl</i> II*	<i>Sal</i> I*	<i>Xho</i> I*
40	4.7	9.6	21.0
41	7.0	11.1	13.0
42	10.5	11.1	8.8
43	9.4	3.6	5.2
44	7.6	3.7	2.8
45	9.4	9.2	15.0
46	7.7	3.8	2.8
47	6.6	7.9	2.8
48	5.4	7.9	2.8
49	4.9	11.1	11.5
50	10.1	8.9	15.0
51	9.8	11.0	+
52	5.4	9.3	+
53	9.8	9.4	+
54	7.6	8.3	+
55	7.8	3.6	+
56	6.6	7.2	+
57	9.8	11.0	+
58	9.0	+	+
59	+	3.6	2.7
60	7.8	3.5	2.7
61	5.0	+	+
62	9.4	10.0	12.2
63	8.4	4.0	+
64	7.4	3.5	2.7
65	5.0	9.2	6.2
66	7.4	+	5.1
67	7.0	10.8	13.0
68	9.1	11.0	+
69	7.6	3.7	2.7
70	7.6	3.7	+
71	9.6	9.8	12.9
72	9.6	10.0	12.9
73	9.6	+	12.9
74	8.1	8.8	+
75	9.7	9.8	14.0
76	3.7	9.9	+
77	5.0	9.1	+
78	+	+	+
79	8.1	4.1	+
80	3.9	+	+
81	6.8	8.0	+
82	7.1	3.8	+

Strain	<i>Bgl</i> II*	<i>Sal</i> I*	<i>Xho</i> I*
83	7.8	4.2	+
84	8.4	+	12.5
85	6.9	3.6	2.7
86	11.3	+	+
87	4.7	8.9	6.2
88	4.9	7.3	2.7
89	+	+	+
90	7.4	8.6	11.1
91	5.2	9.1	6.1
92	8.1	6.7	6.1
93	+	11.5	2.6
94	7.6	+	5.1
95	9.2	10.8	12.1
96	8.6	2.9	+
97	7.6	3.6	2.7
98	9.2	10.1	13.0
99	5.4	12.0	16.0
100	14.0	+	26.0
101	8.6	4.0	+
102	5.2	9.4	6.3
103	6.9	3.9	2.7
104	7.6	3.9	2.7
105	8.6	4.2	6.3
106	5.0	9.4	+
107	5.2	12.5	+
108	9.2	11.5	13.0
109	7.6	4.0	2.7
110	8.2	9.8	11.5
111	8.2	9.8	20.0
112	7.6	4.0	5.3
113	3.8	11.0	8.1
114	7.6	3.8	2.7
115	7.6	3.8	2.7
116	5.0	9.6	+
117	4.7	11.0	14.0
118	4.9	9.6	6.3
119	7.4	3.8	5.0
120	10.1	11.1	8.4
121	8.0	9.1	18.0
122	7.4	+	5.1
123	5.0	9.8	6.4
124	7.8	9.4	11.8
125	8.8	10.2	12.6

Strain	<i>Bgl</i> II*	<i>Sal</i> I*	<i>Xho</i> I*
126	4.9	9.8	6.4
127	9.6	10.8	14.0
128	9.3	10.2	12.6
129	8.2	9.4	8.4
130	8.0	4.1	6.1
131	7.2	3.8	2.6
132	7.2	3.8	2.6
133	4.9	7.6	2.6
134	8.0	4.1	6.0
135	7.4	4.1	6.0
136	4.9	9.8	6.1
137	7.2	3.8	2.6
138	9.8	11.8	8.9
139	7.8	9.2	11.0
140	8.5	3.7	2.7
141	10.1	11.5	8.8
142	7.8	9.1	11.0
143	+	+	2.8
144	7.2	3.7	2.5
145	8.9	9.8	11.8
146	8.4	4.0	6.2
147	+	3.7	2.8
148	+	3.7	2.8
149	7.4	3.7	2.8
150	7.8	9.5	12.2
151	10.5	11.7	9.4
152	7.4	2.7	18.0
153	8.4	4.0	6.4
154	8.4	4.7	6.4
155	7.4	3.7	2.8
156	8.4	4.0	6.4
157	9.1	10.8	13.5
158	5.0	10.0	6.4
159	8.4	4.0	6.4
160	9.1	11.1	13.5
161	7.4	3.9	5.4
162	6.8	3.9	5.4
163	8.1	9.8	11.5
164	9.1	9.1	15.5
165	9.1	9.1	15.5

Strain	<i>Bgl</i> II*	<i>Sal</i> I*	<i>Xho</i> I*
166	4.7	11.1	11.8
167	9.1	11.1	+
168	9.8	10.5	13.5
169	8.4	4.0	6.1
170	7.4	3.8	2.7
171	10.1	11.2	8.6
172	8.1	4.0	6.0
173	4.9	9.6	6.1
174	4.9	6.8	6.1
175	9.0	10.1	12.2
176	7.1	11.3	13.7
177	14.0	9.4	24.5
178	7.4	3.8	2.6
179	10.5	11.5	9.2
180	9.6	10.1	12.9
181	7.4	3.8	2.6
182	8.6	11.0	8.2
183	+	3.5	2.7
184	11.0	12.0	7.2
185	+	+	6.0
186	11.8	10.5	+
187	+	+	2.1
188	5.0	7.6	2.6
189	4.5	9.4	+
190	4.5	9.4	+
191	7.3	3.8	2.6
192	8.5	3.8	2.6
193	8.1	4.1	6.0
194	8.6	10.5	8.9
195	+	+	+
196	7.1	3.8	2.6
197	7.0	10.5	13.7
198	4.8	9.7	9.1
199	9.2	8.5	16.0
200	8.6	2.9	6.0
201	7.7	3.6	5.2
202	7.7	3.6	2.7

Strain	<i>Bgl</i> II*	<i>Sal</i> I*	<i>Xho</i> I*
203	5.0	3.6	+
204	11.1	3.6	+
205	10.1	10.5	14.5
206	8.8	3.6	2.7
207	11.4	+	+
208	+	3.6	+
209	5.0	3.6	+
210	8.6	9.1	8.4
211	10.5	+	7.6
212	8.4	4.0	5.9
213	8.1	9.3	11.8
214	5.0	9.7	6.1
215	10.5	11.7	9.4
216	+	+	+
217	4.9	6.7	6.1
218	8.6	3.5	2.4
219	7.4	3.5	4.9
220	+	+	+
221	9.0	+	15.5
222	+	+	+
223	6.5	3.4	5.1

* Molecular weight in Kilobases (Kb) of probe reactive fragment in Southern hybridization using *Pst*I-*Nru*I and *Pst*I-*Eco*RI probes (see methods).

+ Indicates incomplete data.

Table 2. *P. aeruginosa* with Undistinguishable Probe Reactive Fragments

Strain	<i>Xho</i> I*	<i>Sal</i> I*	<i>Bgl</i> II*
131	2.6	3.8	7.2
132	2.6	3.8	7.2
137	2.6	3.8	7.2
191	2.6	3.8	7.3
178	2.6	3.8	7.4
181	2.6	3.8	7.4
170	2.7	3.8	7.4
133	2.6	7.6	4.9
188	2.6	7.6	4.9
97	2.7	3.6	7.6
202	2.7	3.6	7.7
69	2.7	3.7	7.6
44	2.8	3.7	7.6
114	2.7	3.8	7.6
115	2.7	3.8	7.6
104	2.7	3.9	7.6
109	2.7	4.0	7.6
64	2.7	3.5	7.4
32	2.8	3.5	7.4
2	2.8	3.6	7.4
149	2.8	3.7	7.4
155	2.8	3.7	7.4
134	6.0	4.1	8.0
193	6.1	4.1	8.0
130	6.0	4.1	8.1
11	6.1	4.0	8.1
12	6.1	4.0	8.1
13	6.1	4.0	8.1
172	6.0	4.0	8.1
217	6.1	6.7	4.9
174	6.1	6.8	4.9
16	6.2	3.9	8.5
19	6.2	3.9	8.5
35	6.2	4.0	8.5
36	6.2	4.0	8.5
146	6.2	4.0	8.5
169	6.2	4.0	8.5
25	6.2	4.1	8.8
6	6.3	10.10	5.0

Strain	<i>Xho</i> I*	<i>Sal</i> I*	<i>Bgl</i> II*
1	6.3	10.10	5.1
153	6.4	4.0	8.4
156	6.4	4.0	8.4
159	6.4	4.0	8.4
37	6.4	9.6	4.9
17	6.4	9.6	5.0
151	9.4	11.7	10.5
215	9.4	11.7	10.5
142	11.0	9.10	7.8
139	11.0	9.2	7.8
163	11.5	9.8	8.1
110	11.5	9.8	8.2
123	11.8	9.4	7.8
124	11.8	9.4	7.8
72	12.9	10.0	9.6
180	12.9	10.1	9.6
164	15.5	9.1	9.1
165	15.5	9.1	9.1

*Molecular weight in Kilobases (Kb) of probe reactive fragment in Southern hybridization using *Pst*I-*Nru*I and *Pst*I-*Eco*RI probes (see methods).

Double border separates the different sets of undistinguishable strains.

Discussion

A valid epidemiological marker should possess three characteristics in order to efficiently distinguish amongst strains of *P. aeruginosa* (15). It should be sufficiently sensitive to distinguish unrelated strains, specifically identify all related strains, and needs sufficient stability so that identification of strains does not vary under the usual laboratory conditions or during infection. None of the commonly accepted typing methods have been able to fulfill all these criteria. Some methods are strong in one area and weak in the others. For this reason comparison studies have taken place in order to establish a reliable method, or possibly a combination of methods in order to fulfill the criteria. For example, Meitert & Meitert (14) proposed that the result of O-serological grouping and phage typing of *P. aeruginosa* should be interpreted in a hierarchical way. On this basis, separation according to O groups forms the primary division, and phage typing patterns are used to distinguish between strains of the same O group. Ashehrov (1) and Parker (17) adopted this system, and from the results of extensive studies on the reproducibility and discrimination achieved, concluded that strains of the same O group could only be considered distinct if three or more strong reaction-differences in phage-typing pattern were observed. Other combinations used hierarchically; O serology and pyocin typing, or pyocin and phage typing have been used by investigators with varying success (4). As more typing methods were developed investigators were able to compare more than two typing methods. For example, an article discussing a nursery outbreak of *P. aeruginosa* reviewed epidemiological conclusions from five different typing methods, pyocin sensitivity, serological agglutination of somatic antigens, antibiotic susceptibilities and phenotypic properties (20). In this case only two of these techniques gave results consistent with the patient's history. Another article compares other methods of typing (3). Pitt discusses the advantages and disadvantages of using bacteriocin typing, O-serological typing, H-serological typing, bacteriophage typing, biotyping and antibiograms. He did not conclude that one method was superior, but rather that two or more methods should be used in

collaboration. New methods using DNA have since been developed creating the need for another comparison investigation (9,21,18,26).

Genome fingerprinting has been used to identify different strains of *P. aeruginosa*. Using this typing method chromosomal DNA is digested with restriction endonucleases that cut only rarely, and the large fragments thus obtained are subsequently separated by field inversion gel electrophoresis. The pattern of restriction fragments is characteristic for each strain and provides an estimate of genomic relationship among the strains (9). Fingerprinting offers the advantage of classifying *P. aeruginosa* strains in terms of DNA relatedness. The disadvantages of the method include its cumbersome procedure as well as its difficulty in comparing the strains on a day to day basis.

A more reliable, but perhaps less sensitive method of typing is pilin gene probing. Comparison of primary sequences from several unrelated *P. aeruginosa* strains revealed extensive regions of variability in the carboxy terminal portions (21). The variability of the C-terminal region of pilin is reflected in variable DNA sequence encoding this region. Presence or absence of specific restriction enzyme recognition sites in the vicinity of this region will result in restriction fragment heterogeneity detectable by Southern hybridization. The use of this method is valuable in the sense that it is highly reproducible and it has virtually 0% un-typable strains since no natural occurring strains lacking the pilin gene have been reported. However, this method is not as valuable as other methods in discriminating between different strains of *P. aeruginosa*. For example, only five unique pilin genes among fifteen isolates were characterized, while twelve distinct fragment patterns were detected using the exotoxin A probe with these 15 isolates (18).

The exotoxin A probe method is quite similar to the pilin gene probe method. During Southern hybridization experiments using cloned gene probes encoding the exotoxin A gene and/or surrounding sequences, restriction fragment heterogeneity was observed in the region upstream from the structural gene (26). Later experiments were successful in applying a *PstI-NruI* fragments derived from this hypervariable genomic site as a probe in epidemiological investigations

of *P. aeruginosa* . The DNA probe is capable of distinguishing 90% of the tested strains with only three restriction enzymes. This method proves to be very reproducible, as well as discriminatory among strains of *P. aeruginosa* . Our lab has determined 99 different strains using this method (J. Ogle, unpublished data). Typability is also relatively high using this procedure; approximately 5% of the isolates lack the exotoxin A structural gene and therefore are non-typable with the exotoxin A probes (26). Like the other methods, typing using the exotoxin A probe has disadvantages.

One of the problems occurs when trying to determine whether any two isolates represent truly independent strains. In this case it may be necessary to carry-out a number of restriction digestions of DNA, since the probe-reactive fragments generated by restriction with any one enzyme may appear identical due to limited resolving capabilities of standard agarose gels. Another complication is found in the actual measuring of the fragments. Due to human error it may be necessary to repeat the sample running the digests side-by-side on the same gel.

From the results we have obtained the following conclusions can be reached. There are at least seventeen groups of undistinguishable strains out of 223 isolates completed thus far. The epidemiological strain history is still unknown, as the code has not been broken and, therefore one can only estimate that the strains grouped together were either the same isolates, subsequent isolates taken from the same patient, isolates taken from siblings, or isolates within a limited geographical location. If the undistinguishable strains were in fact taken from different patients who were within a limited area one could conclude that the patients were either cross infected or that they obtained the *P. aeruginosa* from the same source.

Other conclusions can be drawn upon the completion of the study. When all of the results are known comparisons among typing methods may be made. From this information, a possible standard method or combination of two methods can be set. The determination of a standard method will allow epidemiological, and other questions to be studied, and possibly answered.

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